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ORIGINAL ARTICLE

Cell viability study of thermo-responsive core–shell superparamagnetic nanoparticles for multimodal cancer therapy

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Abstract This is a vital extension of our previously published work. Thermo-responsive copolymer coated superparamagnetic $MnFe_2O_4$ nanoparticles are tested for cell viability and affinity on HeLa carcinoma cells under different conditions. Nanoparticles were loaded with anti-cancer drug doxorubicin. Composite nanoparticles of average diameter 45 nm were of core–shell structure having magnetic core of about 18 nm. Magnetic hyperthermia effects on cell viability and drug delivery were studied by exposing the cell suspension to high frequency magnetic field, and living cells were quantified using MTT method. There was almost absence of drug release at 37 °C. Drug was released at temperatures above lower critical solution temperature (LCST) by magnetic heating. LCST of the thermo-responsive copolymer was observed to be around 39 °C. Below this temperature, copolymer was hydrophilic and swelled. But above LCST, copolymer could become hydrophobic, expel water and drug and shrink in volume. Combination of hyperthermia and drug delivery effectively treated cancer cells.

Keywords Biomaterials · Magnetic materials · Nanostructures · Polymers

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Introduction

There is a lot of research going on the biomedical applications of magnetic nanoparticles (MNP) like magnetic resonance imaging, cell separation, localized hyperthermia of cancer and controlled drug delivery. Appropriate biocompatibility of materials is very important, and in this regards iron oxide based magnetic nanoparticles are of key significance. Magnetic field gradient or any other efficient drug delivery system can be used for delivering particles to the tumor sites. When subjected to high frequency magnetic field, these nanoparticles generate heat due to Brownian, Néel and hysteresis losses (Shah et al. 2012). Around a temperature of 45 °C, the healthy body cells remain safe but the cancer cells become vulnerable to radiotherapy and chemotherapy and might even perish completely (Shah 2012; Shah et al. 2010a, b, 2011). This is the approximate temperature that results in mechanisms like protein denaturation, DNA cross linking and apoptosis; all are different cancer damaging mechanisms (Hildebrandt et al. 2002; Kumar and Mohammad 2011; Santos-Marques et al. 2006; Suto and Srivastava 1995). Tissue characteristics, magnetic heater parameters and particles concentration are those factors that influence the effectiveness of localized hyperthermia (Kumar and Mohammad 2011; Raaphorst et al. 1979; Shah et al. 2010a).

The most demanding aspect of MNP is the tailoring of multifunctional characteristics. Core–shell nanostructure is of the utmost importance in which MNP serve as magnetic cores. Opsonization and agglomeration are unwanted in such applications and could be prevented by functionalizing the surfaces of nanoparticles with functional polymers and/or surfactant layers that enhance the biocompatibility and act as shells over cores (Gupta and Gupta 2005; Luong et al. 2011; Martín-Saavedra et al. 2010). Anti-cancer drug can be attached to these particles to deliver to the cancer regions (Derfus et al. 2007; Kumar and Mohammad 2011). Localized hyperthermia and controlled drug delivery are two of the most pivotal purposes served simultaneously by the core–shell nanoparticles.

Another important set of compounds that have attracted huge attention in biomedical research are the functional substances that respond to external stimuli such as pH and temperature, etc. Thermo-responsive polymer PNIPAm (poly-n-isopropylacrylamide) is one of such functional compounds. It has an interesting characteristic of LCST (lower critical solution temperature). Below LCST, it absorbs water and swells in volume due to being hydrophilic. Above the temperature it expels water due to being hydrophobic and so shrinks in volume (Bromberg and Ron 1998; Hoffman 2002; Purushotham et al. 2009; SCHILD 1992; Schmaljohann 2006). By adjusting the temperature of the polymer shell, drug release can be manipulated resulting in higher release rates above LCST and vice versa (Coughlan et al. 2004; Liu et al. 2005; Purushotham et al. 2009; Zhang and Misra 2007). Co-monomer units such as acrylamide could be used to tune the LCST of PNIPAm (Sershen et al. 2000).

Present work is an in vitro study of multifunctional $MnFe_2O_4$ nanoparticles surface-modified with bilayer oleic acid and coated with a thermo-responsive copolymer PNIPAm-co-Am for controlled drug delivery and hyperthermia applications. Cell affinity and viability are studied on HeLa carcinoma cells by MTT method. Figure 1 shows the schematic overview of the processes involved in this study.

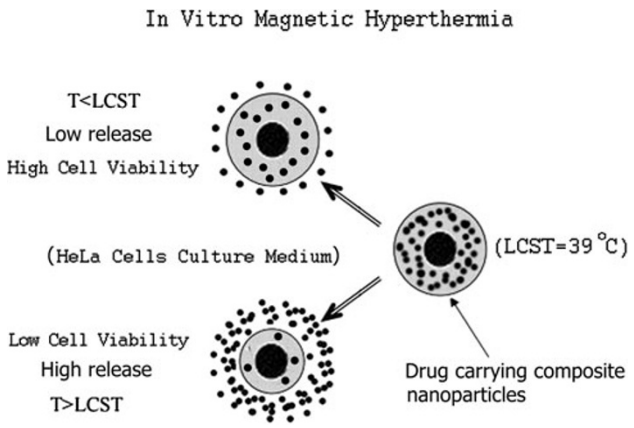

Fig. 1 Schematic overview of the processes involved in this study

Experimental

Current study is the vital extension of our previously published work (Shah et al. 2012). Bilayer oleic acid-modified $MnFe_2O_4$ magnetic nanoparticles (OA-MNP) were prepared by co-precipitation method and coated with PNIPAm-co-Am by emulsion polymerization. Anti-cancer drug doxorubicin (DOX) was loaded into the copolymer chains at 25 °C. Detailed synthesis techniques of composite nanoparticles (CNP) are described in our previous work (Shah et al. 2012).

In vitro study

Magnetic hyperthermia and drug release

The in vitro study of composite magnetic nanoparticles was carried out on HeLa carcinoma cells. Cell viability/cytotoxicity of nanoparticles was determined using MTT method. MTT is a yellow, hydrophilic tetrazolium compound. When this dye is added to the metabolically active cells, it is converted into hydrophobic dark blue formazan due to cleavage reduction of tetrazolium (Kim et al. 2009). Formazan crystals are then dissolved in an organic compound dimethylsulfoxide (DMSO) forming a colored solution and then quantified by finding absorption at 570 nm. The resultant value corresponds to the number of metabolically active cells. 30 mg CNP were added to HeLa cells having concentration of 10^5 cells/ml and incubated for 24 h at an atmosphere of 5 % CO_2 and 37 °C, to allow the nanoparticles to bind to cells. After 24 h, suspension was placed in a thermally insulating glass container and placed within the heating coil of a magnetic heater operating at 7.2 kA/m magnetic field and 70 kHz frequency. Magnetic heater was operated such that 45 °C temperature was maintained for 2, 4 and 6 h for three types of observations. After hyperthermia treatments, suspension was placed in a 96-well plate and incubated for 12 h. Subsequently 20 µl of 5 mg/ml MTT was added to each well and incubated for 4 h at 5 % CO_2 and 37 °C. Medium was removed after 4 h and crystals of formazan were mixed in 200 µl of DMSO. The solution was vigorously mixed to dissolve the reactants. Microplate reader (AMP Platos R-496) was used at 570 nm to determine the absorbance of each well. The relative cell viability percentage was determined by comparing with the control-wells without the magnetic nanoparticles.

Cells affinity test

CNP (30 mg) were added to cell suspension of density 10^5 cells/ml and incubated for 24 h at 5 % CO_2 and 37 °C to allow the nanoparticles to bind to cells. After 24 h, magnetically bound cells were separated by magnetic decantation. Supernatant was processed with MTT assay to count the number of living cells after separating the magnetic cells. Cell affinity was determined by subtracting from the total number of cells.

Results and discussions

There are two reasons why the surface modification of MNP with OA was necessary as reported in last paper (Shah et al. 2012). First, it was to synchronize the attachment of the thermo-responsive copolymer to the MNP by acting as an adhesive providing affinity. Second, was to be used as a surfactant to curb the agglomeration existing because of hydrophobic nature of $MnFe_2O_4$ and the dipole–dipole interactions. Size of nanoparticles is of key significance for biomedical applications. The most appropriate size range of composite nanoparticles is about 10–50 nm for drug delivery and hyperthermia applications (Purushotham et al. 2009). The average diameter of CNP in this work was about 45 nm, of which 18 nm was the diameter of magnetic core. About 60 % (wt) of the (hydrated) CNP was composed of water and organic compounds and the remaining consisted of $MnFe_2O_4$ core. Magnetic core was of pseudo single domain structure and exhibited superparamagnetism due to lack of coercive force (Shah et al. 2012). 'Lower critical solution temperature (LCST) of thermo-responsive copolymer was observed to be 39 °C. Below this temperature, copolymer was hydrophilic and above this temperature, it became hydrophobic and expelled water and drug.

In vitro study on HeLa carcinoma cells

Cells affinity

Composite nanoparticles were added to the cell culture medium and incubated for 24 h to allow the nanoparticles to attach with the cells. Figure 2 shows the cell capture rates of MNP, OA-MNP and CNP determined by MTT method. About 79 % of the cells captured nanoparticles suggesting the appropriate cell affinities of CNP. MNP showed only 20 % affinity to the cells in 24 h indicating their passivity in attaching with the cells in uncoated form. OA-modified nanoparticles showed capture rate of about 47 % cells. The rising of the cells affinity was due to the biocompatible layers coated onto the particles. The attachment with the cells probably occurred due to the CH_3^+ functional groups of PNIPAm-co-Am copolymer that might have interacted electrostatically with the negatively charged glycocalyx on the surface of cancer cells. Cancer cells are observed to be having a high density of negative charge on their surfaces due to the sialic acid residues and glycosaminoglycans on the cell membranes (Kim et al. 2009).

In vitro magnetic hyperthermia

Magnetic hyperthermia characteristics of OA-MNP and CNP were studied by incubating nanoparticles with cells in culture medium for 24 h and then subjecting the suspension to alternating magnetic field of 7.2 kA/m and frequency 70 kHz for 1 h. Temperature profile with time of magnetic heating is shown in Fig. 3. For both observations initial temperature was 25 °C. For 60 min magnetic heating, CNP caused a temperature increase of about 47 °C while the temperature of the suspension containing OA-MNP increased to about 67 °C. It took 35 min for the temperature of the CNP to rise up to 45 °C, which is an appropriate and effective temperature for hyperthermia therapy. Various cellular damaging processes may occur at this temperature like DNA cross linking, apoptosis and protein denaturation (Hildebrandt et al. 2002; Kumar and Mohammad 2011; Santos-Marques et al. 2006; Shah 2012; Suto and Srivastava 1995). By adjusting more suitable magnetic parameters like frequency and strength and the concentration of magnetic particles, the magnetic heating can be further optimized. As described earlier, size of the magnetic core is about 18–20 nm that forms a pseudo single domain structure. Particles exhibit superparamagnetism due to the absence of remanence magnetization and coercive force and heating occurs due to Brownian and Néel losses. Swiftly changing direction of the magnetic moments along the applied field within the crystal lattice

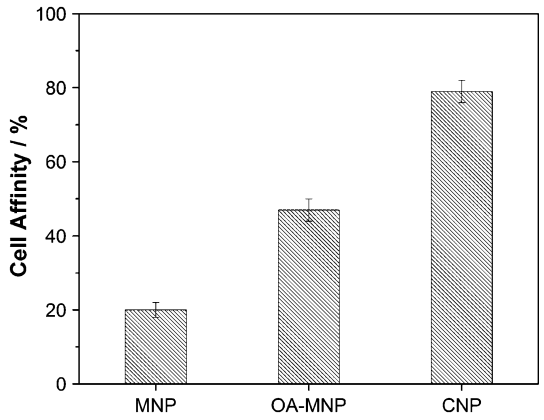

Fig. 2 Cell affinity of MNP, OA-MNP and CNP

(internal dynamics) causes Néel relaxations. The torque of magnetic moments under the alternating field is hindered by the anisotropy energy (that attempts to align the moments along a particular axis within the crystal lattice). After the removal of applied field a reversible process of the relaxation of moments takes place toward their equilibrium position. Energy is dissipated in this process resulting in Néel heating. Under an alternating field, there also exists a physical rotation of the magnetic particles within the fluid medium which causes Brownian heating (external dynamics). Viscosity of the fluid is a determinant for Brownian relaxations. Lesser the viscosity of the medium, greater will be the Brownian heat loss and vice versa.

Cell viability

Cell viability/cytotoxicity of composite nanoparticles was studied using MTT assay on HeLa cells. Nanoparticles were incubated with cells in culture medium for 24 h. Then suspensions were placed at 33, 37 and 41 °C for 6 h for different observations and then incubated again for 12 h at 37 °C. Figure 4 shows the cell viability because of DOX release only in the absence of magnetic hyperthermia. Cell viability was almost maximum for temperatures below LCST where there was marginal DOX release due to diffusion process and copolymer was hydrophilic holding drug molecules in its chains. However, at 41 °C temperature exceeded LCST, copolymer became hydrophobic and expelled DOX that significantly reduced the cell viability to about 63 % due to its anti-cancer effects. There was almost absence of drug release at 37 °C which is significant because CNP holds drug molecules during their transportation through the blood capillaries, and drug will be released to the surrounding only when temperature is made to increase above LCST by magnetic hyperthermia.

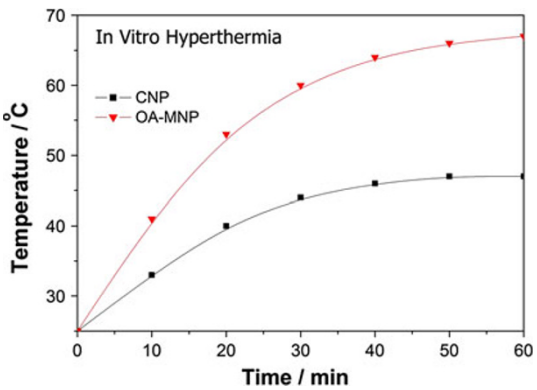

Fig. 3 In vitro heat generation of OA-MNP and CNP under alternating magnetic field

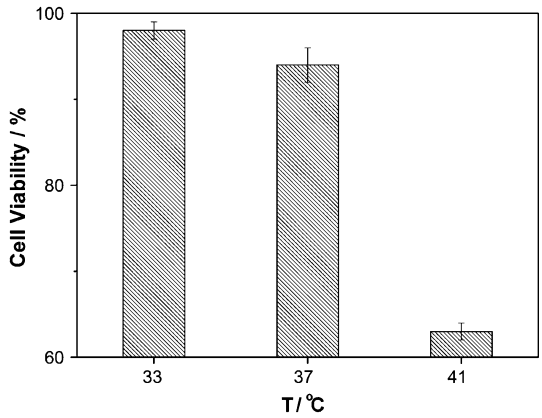

Fig. 4 Cell viability in the absence of magnetic heating (CNP (hydrated) were incubated with cells in culture medium for 24 h. Then suspensions were placed at 33, 37 and 41 °C for 6 h for different observations and then incubated again for 12 h at 37 °C.)

Dehydrated composite nanoparticles (without DOX) were incubated with cells for 24 h and then suspensions were subjected to alternating magnetic field of 7.2 kA/m and frequency 70 kHz in such a way that temperature 45 °C was maintained for 2, 4 and 6 h for three different observations. After magnetic heating each suspension was incubated for 12 h. Figure 5 shows that keeping cells at 45 °C for hours caused a remarkable decrease in the cell viability and it dropped to about 18 % for 6 h magnetic heating. Same experiment of magnetic heating for 2, 4 and 6 h was repeated with DOX-loaded composite particles. Figure 6 shows that anti-cancer drug duly contributed to kill the cancer cells due to its release at temperatures above LCST and viability dropped to about 8 % for 6 h magnetic heating.

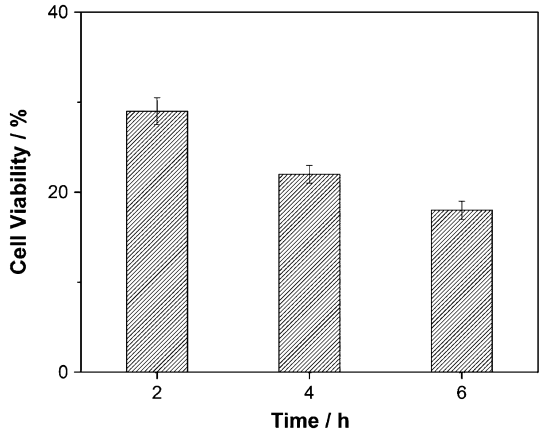

Fig. 5 Cell viability in the presence of magnetic hyperthermia only (dehydrated CNP (without DOX) were incubated with cells for 24 h and then suspensions were subjected to alternating magnetic field of 7.2 kA/m and frequency 70 kHz in such a way that temperature 45 °C was maintained for 2, 4 and 6 h for three different observations)

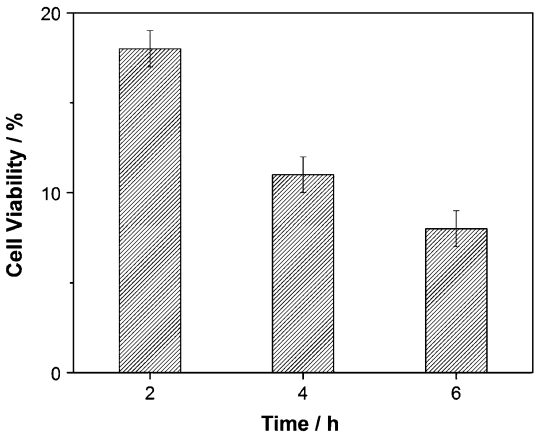

Fig. 6 Cell viability in the presence of magnetic hyperthermia and DOX (CNP (hydrated) were incubated with cells for 24 h and then suspensions were subjected to alternating magnetic field of 7.2 kA/m and frequency 70 kHz in such a way that temperature 45 °C was maintained for 2, 4 and 6 h for three different observations)

Moderate hyperthermia kills cancer cells by apoptosis or at least weakens the cells to enhance the effectiveness of additional procedures like radiotherapy and/or chemotherapy. Therefore, hyperthermia is mostly applied in combinations with other therapies and attaching anti-cancer drug with magnetic nanoparticles in this study is for the purpose of multifunctionality. Intensive hyperthermia may cause cell death due to necrosis in which cytotoxic effect of high temperature causes denaturation and coagulation of membrane and cytoplasmatic proteins (Hildebrandt et al. 2002). It is worth mentioning that in vivo conditions are more favorable than in vitro for hyperthermia because in vivo tumor cells are already under stress due to disorganized vascular system, low oxygen, higher acidic concentration, insufficient nutrients and unfavorable microenvironment. So tumor cells are less able to tolerate the additional stresses of hyperthermia than the healthy cells can do. It is expected that if composite nanoparticles in this work are examined in vivo, even better results may come out.

Conclusion

In vitro study was carried out on HeLa cells for cell affinity and viability under different conditions. Hyperthermia effects on cell viability were studied by exposing the cell suspensions to high frequency magnetic field. There was almost absence of drug release at 37 °C. Drug was released at temperatures above LCST due to magnetic hyperthermia that damaged the cell viability due to the combination of hyperthermia and drug effects. Composite particles exhibited appropriate cell affinity.

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